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Isolation, Purification, and Properties of Cysteine Proteinase from *Bombyx mori* L. Eggs

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Abstract—Silkworm moth (bombyx) egg cysteine proteinase with maximal activity at pH 3.0 was purified by chromatography and isoelectrofocusing. On SDS-electrophoresis in polyacrylamide gel the purified enzyme showed a single band of molecular mass 50 kD. Isoelectrofocusing revealed that the bombyx egg cysteine proteinase exists in two forms with p*I* values of 5.95 and 6.43, respectively. The enzyme activity was sensitive to inhibition by iodoacetamide and *p*-chloromercuribenzoate but resistant to EDTA, pepstatin, and phenylmethylsulfonyl fluoride. The cysteine proteinase hydrolyzes storage proteins of bombyx eggs but it was inactive with respect to N-benzoyl-D,L-arginine-*p*-nitroanilide (BAPNA). It is a cathepsin L-like enzyme.

Key words: cysteine proteinase, eggs, silkworm moth, bombyx, *Bombyx mori* L.

The development of insect embryos is accompanied by large rearrangements of protein bodies during lysis of egg storage proteins and larval tissue formation. This requires both specific peptidyl hydrolases present in eggs and the existence of flexible regulation of their activity; these factors determine the fate of each protein component of the vitellus and the developing embryo [1-4].

We previously demonstrated that a proteinase with pH optimum of 3.0 is maximally present in the proteolytic complex of bombyx eggs over the whole period of embryonal development [5]. According to literature data acidic proteinases play the main role in metabolism of storage proteins during insect embryogenesis [6, 7]. So, in the present study we have isolated and characterized a peptidyl hydrolase with pH optimum of 3.0 from the eggs of the fourth day of bombyx post-diapause development (stage E1, blastokinesis). This stage of bombyx embryogenesis coupled to embryo movement and exhaustion of storage proteins is characterized by high activity of the proteinase with pH optimum of 3.0. So it was important to investigate the physicochemical properties and functional importance of this enzyme in proteolysis of soluble proteins of bombyx eggs.

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MATERIALS AND METHODS

The eggs of M7 × M6 hybrid from Mirgorod egg factory (Ukraine) was used for study of proteolytic enzyme activity of *Bombyx mori* L. Cysteine proteinase was isolated from eggs on the fourth day of post-diapause development. The stage of embryonal development was determined using the method of Mikhailov for microscopic preparation [8].

For protein extraction eggs (1 g) were homogenized in Dounce glass homogenizer in ten volumes of 0.15 M NaCl during 15 min. The homogenate was centrifuged at 10,000*g* for 30 min using a Janetzki K-24 centrifuge (Germany). The supernatant was filtered through a doubled layer of gauze. All these procedures were carried out at 0-4°C.

The resultant supernatant was applied onto a TSK-gel HW-55 column (45 × 1.5 cm) equilibrated with 50 mM Tris-HCl buffer, pH 7.4. The elution rate was 30 ml/h and fraction volume was 3 ml. Protein concentration was measured at 280 nm using a Uvicord flow spectrophotometer (LKB, Sweden).

Fractions possessing proteolytic activity were concentrated using polyethylene glycol (35 kD) and further purified using HPLC with standard LKB equipment. The

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TSK-DEAE-5pW column (21.5 × 150 mm) was equilibrated with 5 mM Tris-HCl buffer, pH 7.4. Proteins were eluted by stepwise NaCl gradient including seven steps: 0, 0.1, 0.2, 0.3, 0.4, 0.5, and 1.0 M.

Preparative isoelectrofocusing [9] was carried out using a Multiphor apparatus (LKB) and Ultrodex gel (LKB). Values of pH in the eluted samples were measured using a pH-150 pH meter (Gomel, Belarus').

Protein content in samples was determined by the method of Lowry et al. [10] using BSA as the standard.

Total proteolytic activity of bombyx egg soluble proteins was determined by the method of Anson [11] modified for assay of peptidyl hydrolase activity in tissues and organs of bombyx [12]. One unit of proteolytic activity of bombyx eggs was defined as the amount of enzyme causing increase in absorbance by 0.01 at 750 nm during 1 min.

Analytic electrophoresis of egg soluble proteins was carried out by the method of Davis [13] modified for insect proteins [14]. Protein sample (up to 200 μg) was applied onto the gel column in 50 μl. SDS-electrophoresis in 7.5% polyacrylamide gel was carried out by the method of Weber and Osborn [15] using 0.05 M Tris-glycine buffer, pH 8.3, containing 0.1% SDS as the electrode buffer.

Substrate specificity of egg cysteine proteinases was studied using the following protein substrates: hemoglobin, casein, azocasein, BSA, azoalbumin, and egg storage proteins isolated by gel-filtration on TSK-HW-55. Protein hydrolysis was evaluated by the method of Anson [11]. The initial protein concentration was 5 mg/ml. The content of egg storage proteins was determined by the method of Lowry et al. [10]. The enzyme activity was also measured using synthetic substrates N-benzoyl-D,L-arginine-*p*-nitroanilide (BAPNA) and succinyl-phenylalanyl-*p*-nitroanilide (SUPHEPA) as described earlier [16]. The enzyme was incubated with 0.5 mM substrate in phosphate-citrate buffer, pH 3.0, at 37°C for 60 min. The reaction was terminated by adding 0.3 ml of 1.5 M CH_3COOH, and the color intensity was read at 405 nm.

RESULTS AND DISCUSSION

Table summarizes data on isolation and purification of proteinase with pH optimum of 3.0 from bombyx eggs.

Figure 1 shows results of fractionation of soluble egg proteins by gel-filtration. Proteinase activity appeared in the zone corresponding to the second and the third peaks. The use of gel filtration in the first stage of purification removed a major part of the extraneous proteins and other contaminants.

Figure 2 shows subsequent separation of protein preparation into several fractions by HPLC. The fraction containing maximal amount of protein possesses proteolytic activity at pH 3.0 and is eluted by 0.2 M NaCl.

The next purification stage employed preparative isoelectrofocusing in an Ultrodex layer. This stage was characterized by significant purification of the enzyme and its separation into two forms with p*I* values of 5.95 and 6.43, respectively (table).

Isolation and purification of proteinase with pH optimum of 3.0 from bombyx eggs

Purification stage	Total protein, mg	Specific activity, U/mg protein	Yield, %
Extract (0.15 M NaCl)	630	0.2	100
Gel-filtration on TSK-HW-55	22.9	6.4	53.6
HPLC on TSK-DEAE-5pW	2.9	25.7	47.8
Preparative isoelectrofocusing on Ultrodex			
Isoform A	0.8	33.9	17.5
Isoform B	0.9	50.0	29.9

For accumulation of an amount of protein sufficient for evaluation of purity of resultant preparations by electrophoresis we carried out seven rounds of isoelectrofocusing. This yielded at least 2 mg of each form of the enzyme. These forms were concentrated using polyethylene glycol (35 kD). SDS-electrophoresis in polyacrylamide gel revealed the presence of a single band of molecular mass 50 kD in each isoform preparation obtained by isoelectrofocusing (Fig. 3a).

Electrophoresis in the absence of SDS revealed that each isoform produced a single band of different mobility. The isoforms with p*I* of 5.95 and 6.43 were characterized by R_f values of 0.40 and 0.45, respectively.

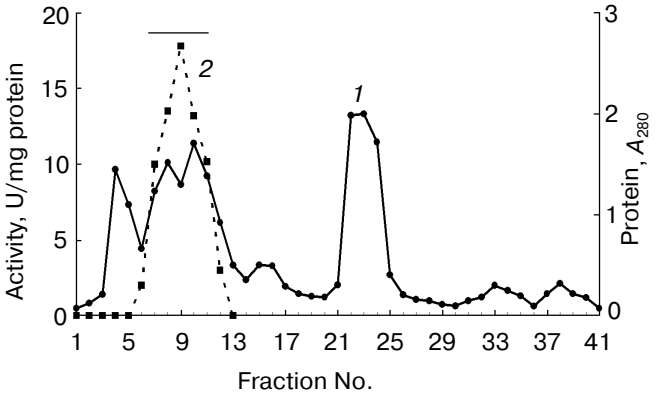

Fig. 1. Gel-filtration of bombyx egg extract on TSK-HW-55 gel column (marked fractions were pooled and used for subsequent purification): *1*) protein; *2*) proteolytic activity.

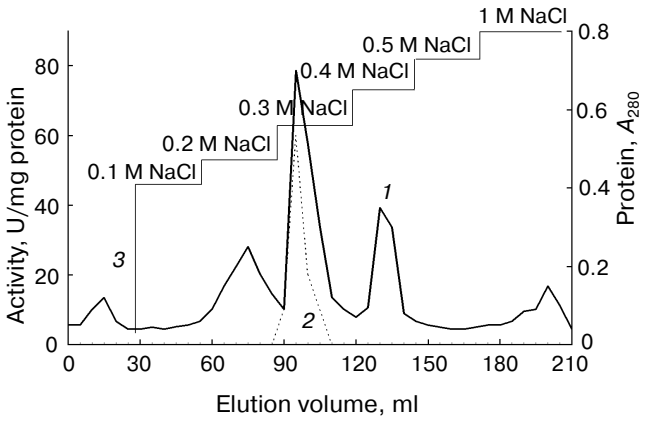

Fig. 2. Proteinase purification by HPLC on TSK-DEAE-5pW (fraction volume 3 ml): *1*) protein; *2*) proteinase activity; *3*) salt concentration.

Determination of molecular mass of the bombyx egg proteinase by gel filtration gave the value of 62 kD (Fig. 3b). This is consistent with molecular masses of insect cysteine [17, 18], serine [19], and aspartyl proteinases [20]. Molecular masses of most insect peptidyl hydrolases vary from 15 to 60 kD.

For subsequent characterization of the purified enzyme we used some inhibitors. The enzyme was preincubated with 1 mM inhibitors in phosphate-citrate buffer, pH 3.0, at 37°C for 15 min. It was markedly inhibited by iodoacetamide and *p*-chloromercuribenzoate (by 60 and 93%, respectively). However, other inhibitors used (EDTA, phenylmethylsulfonyl fluoride, pepstatin) did not inhibit the proteinase activity. These results imply that the purified enzyme is a cysteine proteinase.

Analysis of proteolytic activity during embryonal development of bombyx revealed that increase of cysteine proteinase activity with pH optimum of 3.0 coincided with progressive exhaustion of storage proteins B-1 and B-2 [21, 22] (Fig. 4). These data suggest that a significant amount of B-1 and B-2 observed in the early stages of egg development corresponds to low activity of cysteine proteinases with pH optimum of 3.0. At the end of embryonal development activity of these proteinases significantly increased (by 2.5-fold), and this was accompanied by total exhaustion of storage proteins.

It was interesting to elucidate whether the purified proteinase with pH optimum of 3.0 is active with respect to bombyx egg storage proteins. So, we examined the substrate specificity of the enzyme. The data obtained suggest that the purified proteinase is maximally active with respect to the storage proteins and it is less active with exogenous protein and synthetic substrates.

The purified enzyme effectively hydrolyzed storage proteins B-1 and B-2 to polypeptide fragments (Fig. 5). This is consistent with literature data on hydrolysis of egg storage proteins in other insects [4, 23]. It is known that a cysteine proteinase with pH optimum of 3.6 (a cathepsin B-like proteinase) isolated from bombyx egg hydrolyzed only the storage protein B-2 without any effect on storage protein B-1 [21]. Thus, substrate specificity of cysteine proteinases with respect to storage proteins B-1 and B-2 suggests that proteolysis of soluble enzymes of bombyx egg represents strictly organized and finely regulated process where each enzyme plays its own specific role.

The major proportion of intra-lysosomal proteins is degraded by cathepsins B, D, H, and L. Study of digesting activity of rat liver lysosomes *in vitro* revealed that more than 90% of cellular proteins are degraded by concerted action of cathepsins B, D, and L [24, 25]. Taking into consideration all features of the purified enzyme from bombyx eggs we believe that it should be considered a cathepsin L-like enzyme. Like mammalian cathepsin L the purified insect proteinase is localized in lysosomes; it has acidic pH

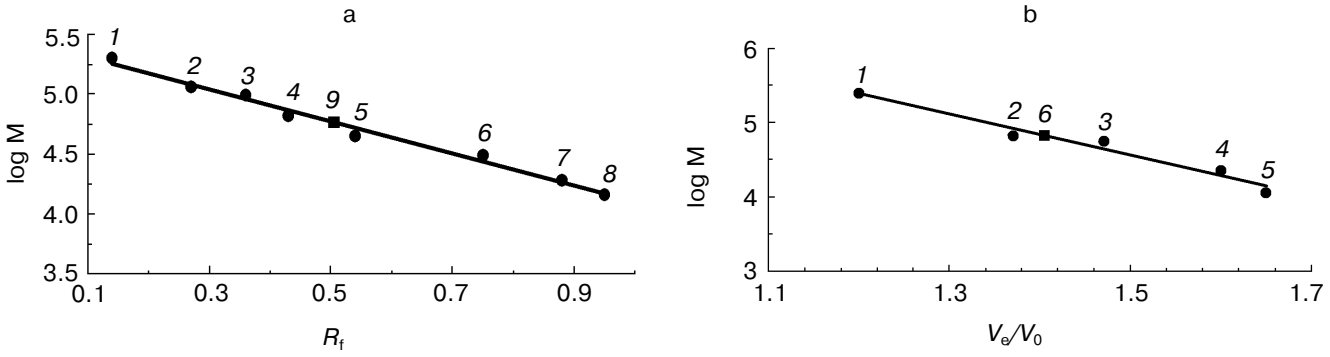

Fig. 3. Determination of molecular mass of the proteinase with pH optimum of 3.0 from bombyx eggs: a) by SDS-electrophoresis in 7.5% polyacrylamide gel (protein markers: *1*) myosin (200 kD); *2*) β-galactosidase (116 kD); *3*) phosphorylase *b* (97 kD); *4*) serum albumin (66 kD); *5*) ovalbumin (45 kD); *6*) carboanhydrase (31 kD); *7*) trypsin inhibitor (21 kD); *8*) lysozyme (14 kD); *9*) proteinase with pH optimum of 3.0); b) by gel-filtration on TSK-HW-55 (protein markers: *1*) catalase (252 kD); *2*) BSA (67 kD); *3*) glutamate dehydrogenase subunit (56 kD); *4*) soybean trypsin inhibitor (22 kD); *5*) cytochrome *c* (12 kD); *6*) proteinase with pH optimum of 3.0).

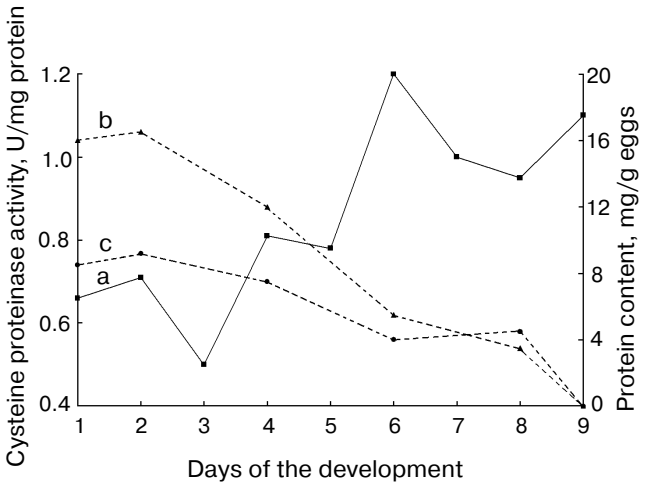

Fig. 4. Change in storage protein content and activity of cysteine proteinase with pH optimum of 3.0 during bombyx embryogenesis: a) proteolytic activity; b) protein B-1 content; c) protein B-2 content. Abscissa shows days of egg development: *1*) unfecundated; *2*) freshly oviposited; 3-4) first, third, and fifth days of pre-diapause development; 5) diapausing eggs; 6-8) second, fourth, sixth, and seventh days of post-diapause development; 9) one-day-old larvae.

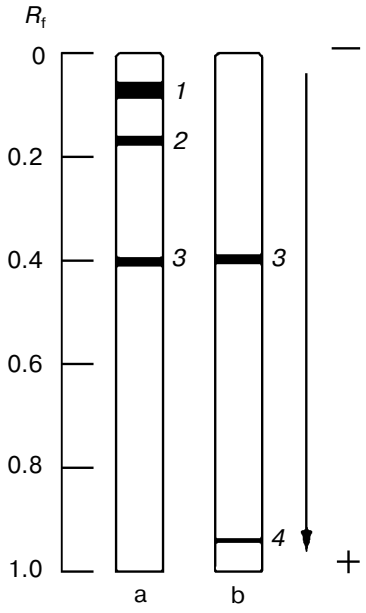

Fig. 5. Electrophoregram of egg storage proteins (in 7.5% polyacrylamide gel) incubated with the purified preparation of cysteine proteinase: a) intact storage proteins (control); b) storage proteins after treatment with proteinase; *1*, *2*) storage proteins; *3*) cysteine proteinase with pH optimum of 3.0; *4*) products of hydrolysis of storage proteins.

optimum. The enzyme is a cysteine proteinases. It is inactive with respect to cathepsin B substrate—BAPNA—but hydrolyzes endogenous storage proteins. The purified enzyme exists as at least two isoforms.

Among insects cathepsin L-like proteinase was found only in eggs of red cockroach [4] and bombyx [21].

Thus, two cysteine proteinases involved in metabolism of storage proteins have been found in bombyx eggs. One of them is a cathepsin L-like protein catalyzing cleavage of storage proteins B-1 and B-2. The second one described by Takahashi et al. is a cathepsin B-like enzyme. The role of this enzyme probably consists in degradation of embryonal vitelline proteins including vitellogenin [2, 21, 26].

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